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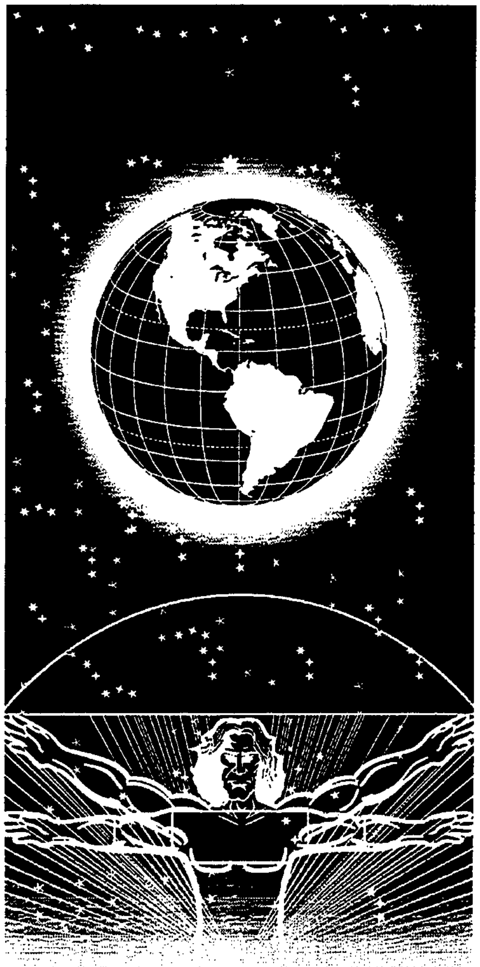

# UNITED STATES AIR FORCE ARMSTRONG LABORATORY

# THE BIOLOGICAL EFFECTS OF ADN ON HEPATOCYTES: AN EPR STUDY


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**FOR THE DIRECTOR**

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**13. ABSTRACT** *(Maximum 200 words)*
This project investigated the biological effects of ammonium dinitramide (ADN) on hepatocytes. It was hypothesized that ADN decomposes to form free radicals which would be deleterious to the body. The effects of ADN on the liver were studied because regardless of the route of exposure, once inside the body it will enter the bloodstream and ultimately pass through the liver. The leakage of the enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) was measured to ascertain the viability of WB 344 hepatocytes after a 24 h exposure to ADN. Electron paramagnetic resonance (EPR) spectroscopy was used to determine if ADN induced the production of free radicals. As free radicals are highly reactive, a-phenyl-tert-butyl nitrone (PBN( and 5,5-dimethyl-1-pyrolline-1-oxide (DMPO) were used to trap the radicals produced in the experiments. Incubation of hepatocytes with 2.8 mM ADN for 24 h was toxic to 50% of the cells. Cells exposed to ADN produced free radicals in the presence of both PBN and DMPO. The generation of free radicals using PBN seemed to be pH dependent. Further studies are necessary to determine the effects of ADN on the possible target organs, the lungs and skin.

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## PREFACE

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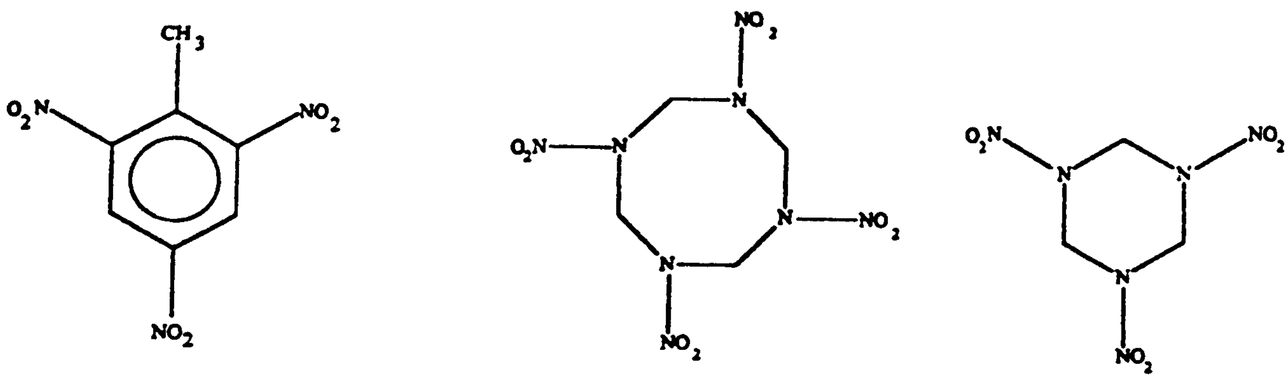
### Introduction

One of the major objectives of occupational health and environmental toxicology research is to determine and quantify risks that may occur as a result of exposure to experimental chemicals (1). These risks must be ascertained as early as possible so that proper safety measures may be taken throughout the research and development of new chemicals. The main goals of studying the biological effects of exposure to the oxidizer ammonium dinitramide (ADN) is to ensure that the current or past exposure of workers is "safe" (ie does not permit an unacceptable health risk) and to detect potential excessive exposure before the occurrence of detectable adverse health effects. It is essentially a preventative medical activity.

The results of this biological effects program could also potentially be used to make a biological monitoring device. Such a device could be used to interpret exposure on an individual basis, which could then be used to estimate for each examined worker the amount of exposure absorbed during a specific time interval or the amount retained in the organism or bound to critical cells in the body or even to soil particles at waste sites. The information may also be used to appreciate the overall work hygiene conditions by analyzing the distribution of the biological results in a group of workers. It evaluates the internal dose received and hence helps to estimate health risks. The greatest advantage of understanding the biological effects of ADN is the fact that the biological

parameter of exposure is more directly related to the adverse health effects which the U.S. Air Force attempts to prevent than any environmental measurement.

ADN is a new type of energetic material (2). Currently, several chemicals including TNT, HMX, RDX, and AP (ammonium perchlorate) are being used as high energy solid fuels, Fig. 1.



TNT HMX RDX

Figure 1. Currently used high energy solid fuels

Recent research is leading to the development of replacements for these compounds. ADN is being investigated as an alternative compound to AP. It is predicted that ADN will be an improvement over AP in weapons systems development or as an oxidizer in the solid fuel of the booster rockets used to put the space shuttle in orbit (2). First, ADN contains no chlorine atoms. Chlorine is a potential pollutant. Second, ADN would not result in the contrail currently produced by AP (2). The absence of this trail will make the detection of rocket launches powered by ADN more difficult. Third, ADN will permit an increased payload capacity which is important for space launches. ADN is an oxide of nitrogen and its chemical formula is $NH_4N(NO_2)_2$ (3). Based on the chemical formula of ADN it can decompose to nitrogen dioxide, $NO_2$ (4). $NO_2$ is a free radical.

Free radicals can be defined as a molecule or ion containing an unpaired electron (5). Free radicals are very reactive and can cause injury to biological tissue (6-10).

ADN has been experimentally shown to produce $NO_2$ on exposure to gamma-radiation (4). Although the possible ADN induced free radical reactions can be shown chemically (Fig. 2), it is not known whether they can occur within living cells.

(1) $2NH_4^{+}N(NO_2)_2 \xrightarrow{\text{radiation}} 2NH_3^{\cdot} + 4NO_2^{\cdot} + 2H^{+} + N_2$

(2) $NH_3^{\cdot} + H^{+} \longrightarrow NH_4^{+}$

(3) $NH_4^{+} + 2O_2 \longrightarrow NO_3^{-} + H_2O + 2H^{+}$

(4) $NH_4^{+} \xrightarrow{\text{Urea Cycle}} CO(H_2N)_2$

(5) $O_2^{\cdot -} + O_2^{\cdot -} + 2H^{+} \longrightarrow 2H_2O_2 + O_2$

(6) $Fe^{+2} + H_2O_2 \longrightarrow Fe^{+3} + \cdot OH + OH^{-}$

(7) $NO_2^{\cdot} + \cdot OH \longrightarrow NO_3^{-} + H^{+}$

(8) $NO_3^{-} + 3Fe^{+2} + 4H^{+} \longrightarrow NO + 3Fe^{+3} + 2H_20_2$

(9) $H_2O_2 + NO_2^{-} + H^{+} \longrightarrow ONOOH + H_2O$

(10) $O_2^{\cdot -} + NO\cdot + H^{+} \longrightarrow ONOOH$

(11) $ONOOH \longrightarrow \cdot OH + NO_2^{\cdot}$

(12) $ONOOH \longrightarrow (ONOOH)^{*} \longrightarrow NO_3^{-} + H^{+}$

Figure 2. Possible pathways for ADN decomposition (Ref. 4)

The best technique to study free radicals is electron paramagnetic resonance spectroscopy (EPR). Radicals in concentrations down to about $10^{-10}$ M can be detected by EPR (11). In this technique a sample placed in a magnetic field is subjected to microwave radiation. The unpaired

electron acting as a magnet can take up two orientations with respect to the external field corresponding to two energy levels. The energy difference between these levels induced by the microwave radiation produces an absorption peak which is detected by the spectrometer (12). As free radicals react very quickly, one way of detecting them is by spin trapping. Spin trapping consists of reacting short-lived free radicals with a spin trap (usually a nitrone or nitroso compound) yielding a longer-lived nitroxide spin adduct which can be detected by EPR (13). There are a number of spin traps which can be used in biological systems (9,13). The most commonly used spin traps are a-phenyl-tert-butyl nitrone (PBN) and 5,5-dimethyl-1-pyrolline-1-oxide (DMPO).

It was hypothesized that the main routes of exposure to ADN would be through the lungs or skin. Study of the biological effects of ADN have to take into consideration absorption by all routes. Regardless of the route of entry of ADN, once inside the body it will enter the bloodstream and will ultimately pass through the liver. The liver is the largest gland in the body (14) and is often the target organ of chemical-induced tissue injury, a fact recognized for over 100 years (15-16). Hazard assessment studies often focus on the liver because it is the organ largely responsible for the detoxification and metabolism of chemicals in the body. While, the biological effects of exposure to ADN in the liver can be studied in many ways, the initial study of the biological effects in cultured hepatocytes is the most logical because it is economical, provides large supplies of samples and requires no animals. The objective of this project was to study the biological effects of ADN on the viability and proliferation of hepatocytes and to measure the free radical decomposition products of ADN by EPR and EPR/spin trapping techniques.

## METHODOLOGY

### Cell Culture

WB 344 hepatocytes were isolated and cultured in DMEM (10% fetal bovine serum, 1% penicillin/streptomyocin, pH 7.4). The cells were allowed to become confluent, and diluted to a concentration of $5 \times 10^5$ cells/mL before use in the cell viability assay.

### Cell Viability

Cell viability was measured on WB 344 hepatocytes to determine the integrity of the cell membrane. Using the Kodak Ektachem 700XR, the leakage of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was ascertained. The lactate dehydrogenase (LDH) assay was conducted on the Dupont ACA discrete clinical analyzer.

### Cell Proliferation

A CellTiter 96 Non-Radioactive Cell Proliferation Assay was conducted using a Molecular Devices Thermo max microplate reader. This assay was used to determine the absorbance of the sample which is directly proportional to the number of viable cells.

### EPR Spectroscopy

Cell preparations ($1 \times 10^6$ cells/ml) were packed in quartz aqueous cells. Using a Varian E4 EPR spectrometer, the EPR spectra were recorded under the following conditions: microwave power, 20 mW; microwave frequency, 9.54 GHz; scan range, 200 G; field set, 3430 G; time constant, 0.5 sec; modulation amplitude, 1 G; and modulation frequency, 100 kHz.

## Results

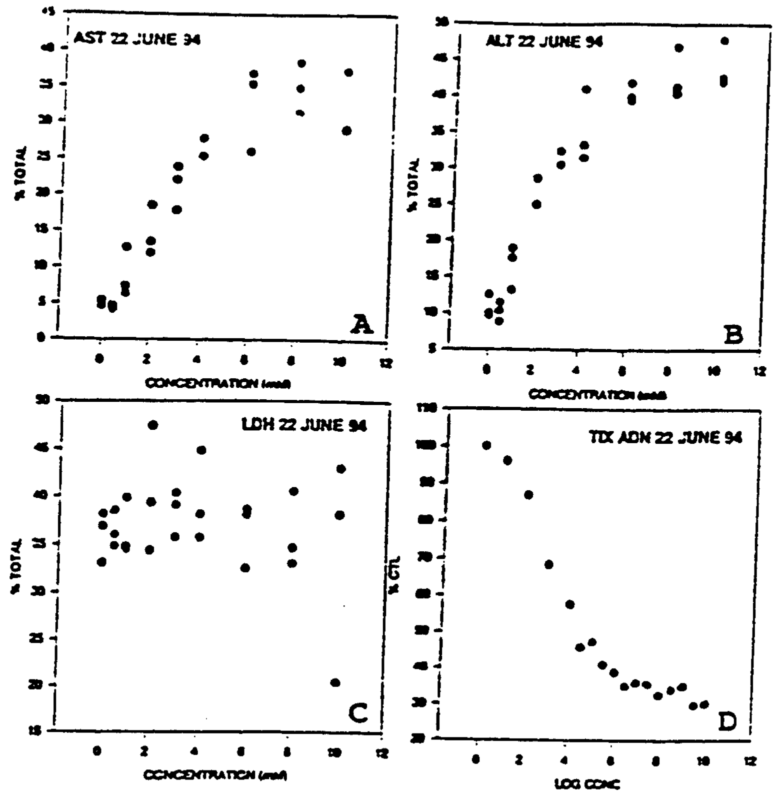



Figure 3. Viability (A-C) and proliferation (D) assay results of WB 344 hepatocytes following a 24 hr. exposure to ADN.

After a 24 hr. exposure to ADN, viability assays were taken to determine the leakage of the enzymes AST, ALT, and LDH. Figures 3A and 3B show the effect of ADN on the leakage of AST and ALT. In both cases, an increase in the ADN concentration is reflected in the greater percentage of enzyme leakage. Figure 3C shows the results of the LDH viability assay. Irrespective of ADN concentration, the assay gave the same LDH leakage value (37±10%). The results of the cell proliferation test are displayed in Figure 3D. As ADN concentration (mM) in the media was increased, there was a decrease in the number of surviving cells.

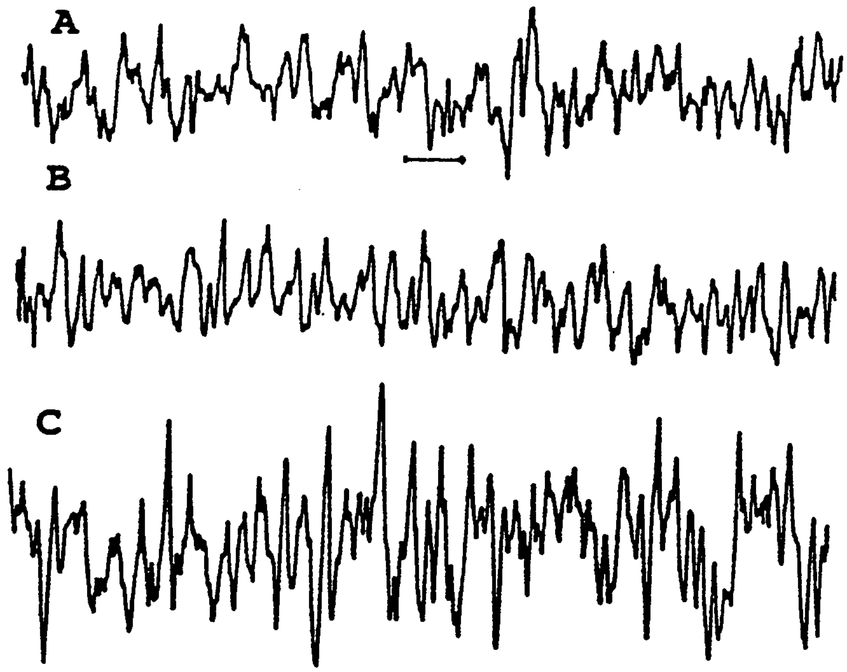



Figure 4. EPR spectra of hepatocytes and PBN (4A), ADN and PBN (4B), and PBN and hepatocytes with ADN (4C).

Figure 4 displays EPR spectra gathered under the following conditions: scan range, 200 G; field set, 3430 G; time constant, 0.5; modulation amplitude, 1 G; receiver gain, 10 x $10^4$, microwave power, 20 mW; and microwave frequency, 9.54 GHz.All tests were conducted at room temperature. Figures 4A and 4B, show the EPR spectra obtained when the spin trap 0.02 M PBN was added to WB344 hepatocytes and incubated for 30 min at 37°C,and when 1 M ADN is added to PBN without cells, respectively. For both of these samples, the spectra represent random noise. Figure 4C displays the EPR spectra produced after the 30 minute incubation of ADN in PBN with cells (1 x $10^6$ cells/mL). In this spectrum, the presence of spin adducts are not clear although their formation is beginning.

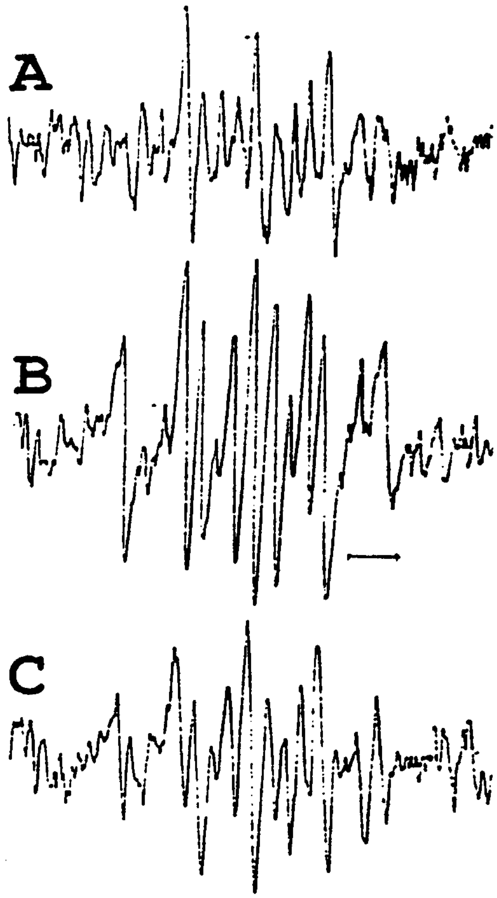



Figure 5. EPR spectra of hepatocytes and DMPO (5A), ADN and DMPO (5B), and cells and DMPO with ADN (5C).

With the exception of receiver gain, the conditions in Figure 5 remained the same as those explained in Figure 4. Figure 5A (receiver gain 1 x $10^4$) shows the EPR spectrum of the spin trap 0.02 M DMPO and WB 344 hepatocytes (1 x $10^6$ cells/mL) after a 30 min. incubation at 37°C. Unlike the spectrum of the cells and spin trap from the previous figure (4A), this figure shows a nitroxide triplet of hyperfine coupling constant $a_N$=15.0. The hyperfine coupling constants were measured directly from the spectra as the separation in peaks measured in mT. Figure 5B (receiver

gain 8 x $10^4$) is the spectrum drawn when ADN and DMPO were tested immediately after mixing. Figure 5B consists of two DMPO adducts. The first consists of a nitroxide triplet with similar hyperfine coupling constants as those described in Figure 5A. The second DMPO spin adduct consists of a triplet of triplets suggesting the addition of a nitrogen center to the DMPO. The hyperfine coupling constant of these spin adducts are $a_N$=12.0 for the primary nitrogen, $a_{Nb}$=5.0 for the secondary nitrogen. Figure 5C is identical to Figure 5B, but less intense, suggesting that the cells compete for the free radicals of ADN in the presence of DMPO.

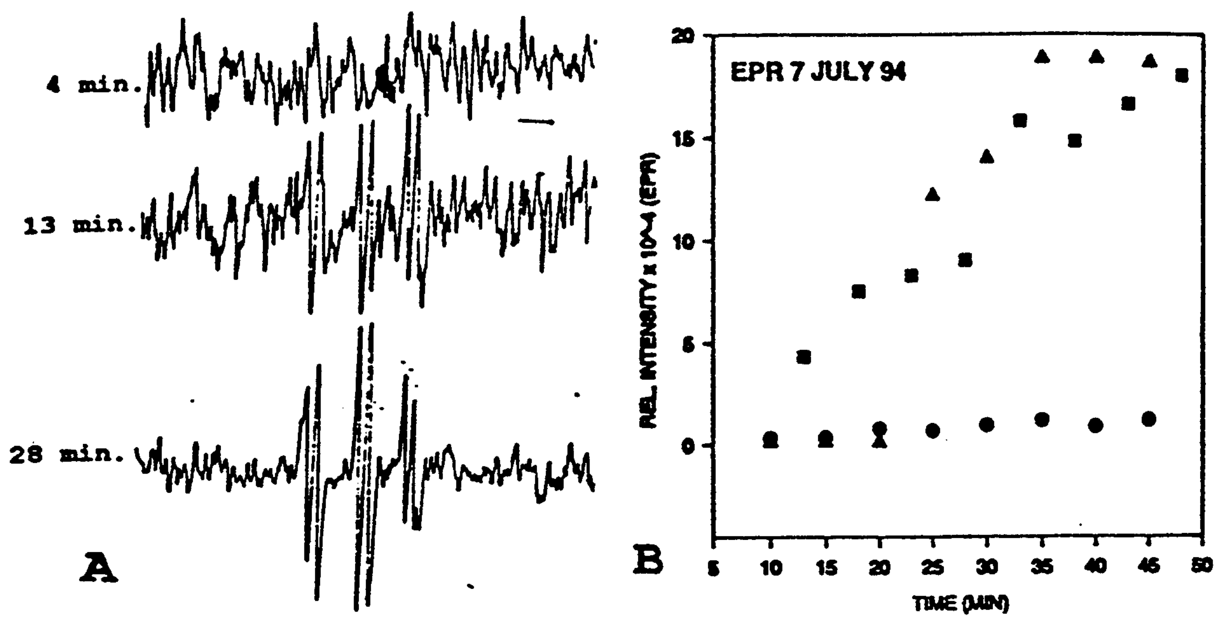



Figure 6. The EPR/spin trapping spectra over time (6A) and (6B) graph of 10 uL NaOH with 1 M ADN in .02 M PBN

Figure 6A shows the spectra from a run in which 10 uL of NaOH was added to .02 M PBN and 1 M ADN. Over time, the peaks increased in intensity. The relative intensity, calculated from the peak height divided by the receiver gain, reached a maximum at $5.64 \times 10^{-4}$. Figure 6B is the graph of the relative intensity of the EPR signal over 50 minutes, for three different concentrations (5.2 uL, 10 uL, and 20 uL) of NaOH in PBN and ADN (pH=8.6, 9.3, and 9.7 respectively). In all cases except the 5.2 uL concentration, the intensity of the EPR signal grew significantly over time.

## Discussion

The effects of ADN on cell viability and free radical production in the liver have been studied using EPR/spin trapping and various viability assays. Based on the literature search, this is the first study on the effects of ADN on hepatocytes. Figure 3 demonstrated the effect of ADN on cell viability as determined by AST, ALT, LDH, and proliferation assays. Three of these four tests showed that increasing concentrations of ADN caused decreased cell viability, while in the LDH assay no trend was visible. Figures 4 and 5 show the EPR spectra gathered under different spin traps. The use of PBN as the spin trap indicated the presence of free radicals only in the sample containing ADN, PBN, and hepatocytes. Peaks were identified in all the samples in which DMPO was used as the spin trap. This is due to the fact that DMPO detects oxygen-centered radicals which are formed naturally in the body, yielding DMPO-adducts with a distinctive characteristic pattern. Figure 6 is the result of an experiment in which 10 uL of NaOH was added to .02 M PBN and 1 M ADN. Over time, the intensity of the peaks grew larger, indicating the growing presence of free radicals as time

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elapsed. The pH of the solution appears to be important in determining free radicals in ADN with the spin trap PBN. All tests involving cells had a pH 6.9 ± 0.29 and the spin adducts formed by ADN were unclear.

The tests conducted in this study (Figure 3D) indicate that ADN is toxic to 50% of cells at a concentration of 2.8 mM. EPR/spin trapping data indicates that production of free radicals occurs in hepatocytes in the presence of ADN, and this production increases over time. Further studies must be conducted with various routes of exposure (eg. inhalation, dermal absorption, and ingestion) in order to determine exposure limits and establish safety standards regarding the use of ADN in the workplace.

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